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Increased production of tumour necrosis factor-α, interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease

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Abstract

***Background*—Increasing evidence points to a important role for inflammatory cytokines for the pathogenesis of Crohn's disease.**

***Aim*—To compare the secretion rate of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) by morphologically normal and inflamed intestinal mucosa from patients with Crohn's disease.**

***Results*—Organ cultures of intestinal biopsy specimens taken from areas of affected mucosa from patients with Crohn's disease spontaneously produced increased amounts of TNF-α, IL-1β, and IL-6 compared with controls but also biopsy specimens taken in macroscopically and microscopically unaffected areas in the same patients. Concentrations of IL-1β and IL-6 measured in the supernatant fluid of biopsy cultures were positively correlated with the degree of tissue involvement measured by both endoscopic and histological grading. By contrast, TNF-α concentrations were not correlated to endoscopic and histological grading.**

***Conclusions*—These consistently raised TNF-α, IL-1β and IL-6 secretions by normal appearing mucosa from patients with Crohn's disease provide evidence for a sustained immune stimulation in Crohn's disease even in the absence of patent inflammation. The results shed a new light on the role of inflammatory cytokines in the onset of intestinal tissue damage in Crohn's disease and suggest that the range of intestinal lesions in Crohn's disease may be wider than suspected on the basis of regular endoscopic and histological examinations.**

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Keywords: Crohn's disease, cytokines, organ cultures, inflammation.

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Several factors have been suggested as possible initiating events in the development of inflammatory bowel disease, including bacterial or viral organisms, dietary products and environmental toxins. These factors are suspected – in genetically prediposed people – to initiate a sequence of chronic immunological processes that perpetuate a prolonged intestinal immune response leading to tissue injury and the subsequent clinical symptoms.

Early recurrence of Crohn's disease after ileal resection and ileocolonic anastomosis in apparently normal intestinal tissue,[1] and the finding of granulomas even in endoscopically normal areas of mucosa,[2] suggest that local synthesis of inflammatory mediators may occur in morphologically normal mucosa of patients with Crohn's disease and that the range of Crohn's disease may be larger than detected by conventional endoscopic and histological examinations. Among the numerous soluble biochemical mediators released by activated intestinal cells, particular attention has been given to macrophage derived inflammatory cytokines. Recent studies of inflamed mucosa from patients with inflammatory bowel disease have identified intestinal activated macrophages as the main producers of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β),[3] as well as the major producers of interleukin-6 (IL-6) in conjunction with epithelial cells.[3–5]

To date, no data on the simultaneous release of TNF-α, IL-1β, and IL-6 by organ cultures from intestinal biopsy specimens from both normal appearing (endoscopically and histologically) and affected intestinal mucosa from patients with Crohn's disease has been considered. We therefore examined the spontaneous release of these three cytokines by macroscopically and microscopically unaffected intestinal mucosa from patients with Crohn's disease, and compared the results with those from affected mucosa from patients with Crohn's disease and normal control intestinal tissue.

Patients and Methods

INTESTINAL BIOPSIES

Thirty five patients with Crohn's disease were prospectively included. The Table shows the clinical data and treatments at the time of the study. The activity of disease was assessed according to Van Hees *et al.*[6] No patient had extraintestinal manifestations of Crohn's disease or extraintestinal infection. Furthermore, serological studies concerning *Yersinia enterocolitica* and cytomegalovirus infection, as well as stool cultures for *Clostridium difficile* were negative in all cases. Small bowel and colonic biopsy specimens were obtained from

Clinical characteristics and treatment at the time of inclusion of the patients with Crohn's disease (CD). The disease activity was based on the Van Hees index[6]

	Patients with CD (n=37)
Age (y) (mean (range))	36 (20–57)
Men/women	18/19
Disease activity (n):	
Severe	10
Moderate	14
Mild	13
Bowel involvement (n):	
Ileum alone	8
Colon alone	10
Ileocolonic disease	19
Current treatment:	
Steroids >10 mg/day	13
Azathioprine+steroids	3
Mesalazine	10
No medication	11

macroscopically and microscopically non-affected (n=26) or affected (n=26) areas of the intestinal mucosa during colonoscopy in patients with Crohn's disease. In 17 patients biopsy specimens were taken in both affected and unaffected mucosa. All patients required a colonoscopy for medical reasons. The control group included 26 patients without inflammatory bowel disease who underwent endoscopy for polyp or colon cancer surveillance (n=8), irritable bowel syndrome (n=12), lower gastrointestinal tract bleeding (n=4), and preoperative controls from female patients with non-neoplastic extra-intestinal pelvic mass (n=2). All controls were free of intestinal disease. In each patient a set of six biopsy specimens was taken, two were fixed in formalin for histological assessment, four were placed at 4°C in a medium consisting of Ca^{2+} and Mg^{2+} free (CMF) Hank's solution (Gibco-BRL, Cergy Pontoise, France), supplemented by 100 IU/ml penicillin and 100 μg/ml streptomycin (Diamant, Puteaux, France).

ENDOSCOPIC GRADING

The severity of inflammation was endoscopically graded according to Wardle *et al.*[7] The four point scale corresponds to: (1) macroscopically normal, (2) granular mucosa, contact bleeding, (3) erythematous and oedematous mucosa, aphtoid or superficial ulceration, (4) deep ulceration with slough, and inflammatory pseudopolyp formation. Biopsy specimens were taken from both inflamed (graded 3 or 4), and quiescent (graded 1) areas. Grade 2 mucosa was not considered for this study to avoid an overlap in biopsy groups.

TISSUE CULTURE

After collection, biopsy specimens were transferred to the laboratory. Within a maximal lag of three hours after biopsy, tissue was gently washed three times in CMF-Hank's medium supplemented by antibiotics, blotted carefully, weighed, and individually placed in 24 well tissue culture plates (1 ml culture medium/well). The culture medium consisted of RPMI-1640 (Gibco) supplemented by 10% heat inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), penicillin (100 IU/ml), and streptomycin (100 μg/ml). After 18 hours' culture at 37°C in a humidified 95% air/5% CO_2 atmosphere, medium was removed, filtered and stored at -80°C until required for cytokine assays.

HISTOLOGY AND HISTOLOGICAL INFLAMMATION SCORING

Biopsy specimens from each patient were fixed in 10% buffered formalin and embedded in paraffin wax after dehydration, clearing, and impregnation. Subsequent 4 μm sections were stained with haematoxylin and eosin. Morphometry and calculation of an inflammation score were performed. The morphometric assessment used a method for the quantitative examination of intestinal mucosal biopsies to determine an index related to the surface to volume ratio of the intestinal mucosa as previously described by Dunnil and Whitehead,[8] and specifically used in inflammatory bowel disease by Dunne *et al.*[9] This technique gave an index of surface area and an index of mucosal volume. All biopsy specimens for histology were assigned an overall inflammation score adapted from Riley *et al.*[10] Tissue examination was performed blindly according to the diagnosis and the endoscopic inflammation score. Sections were graded using five histological features: polymorphonuclear cell infiltrate in the lamina propria, crypt abscesses, surface epithelium integrity, chronic inflammatory cell infiltrate (mononuclear cells in the lamina propria), and crypt architectural integrity. As this study included only Crohn's disease the criterion of mucin depletion used by Riley *et al*[10] was not taken into account. Each histological feature was graded on a four point intensity scale: none (1 point), mild (2 points), moderate (3 points), or severe (4 points); therefore, the overall inflammation score ranged from 5 to 20. Only the biopsy specimens in which the histological score was 5 were used in the control group as well as in the group of normal appearing biopsies from patients with Crohn's disease. However, morphometry showed that in inflamed mucosa from patients with Crohn's disease the surface area was reduced and the mucosal volume was significantly (×1·5) increased compared with normal appearing areas in the patients.

In Crohn's disease, overall (affected and non-affected mucosa) endoscopic and histological grades were positively correlated (r=0·9; $p<0·0001$).

The structural integrity was assessed by standard histology and by measurement of lactate dehydrogenase release according to Wardle *et al.*[7] After a 18 hour culture no histological changes were noted as compared to precultured tissue. Furthermore, the release of lactate dehydrogenase in cultured tissues was significantly lower than in uncultured tissues (data not shown).

IMMUNOASSAYS FOR CYTOKINES

Interleukin production (TNF-α, IL-1β, and IL-6) was measured with specific immunoassays. Each enzyme linked immunosorbent

assay (ELISA) used two monoclonal antibodies directed to two different epitopes on the corresponding interleukin molecule. The monoclonal antibodies were developed using purified recombinant cytokine and do not cross react with a panel of related and non-related cytokines.[11] Quantitative evaluation of interleukins secreted by monocytes was achieved by ELISA using conditions described by Kenney *et al*[11] with minor modifications to improve the sensitivity and specificity: Tween (500 µl/l of phosphate buffered saline) was preferred to thimerosal in the incubation, blocking, and washing buffer. Polyvinyl chloride plates (Costar, No 2596) were coated with 50 µl per well of antibodies (15 µg/ml) and incubated overnight at 4°C. After three washing steps, and the non-specific saturation step, 25 µl standard (human recombinant cytokine) or sample were added to 25 µl secondary biotinylated monoclonal antibody (2 µg/ml) and the mixture incubated for two hours at room temperature. After the washing steps, 50 µl of peroxidase-streptavidin (1:3000 in PBS-Triton) was added and the mixture allowed to stand for one hour at room temperature. A colorimetric reaction (OD at 450 nm) using o-phenylenediamine dihydrochloride as peroxidase substrate was performed. Sensitivity of the assays was below 20 pg/ml and all samples gave values within the working range of the standard curves. Concentrations (pg/ml) of unknown samples were computed by interpolation with a standard curve run on each plate (curve fitting: four parameter logistic). All samples were analysed in duplicate. Results of cytokine concentrations in supernatant fluids were expressed according to the weight of the corresponding biopsy specimen (pg/mg tissue/ml culture medium) and the final result for each patient corresponds to the mean of the four individual biopsy cultures.

STATISTICS

Data are expressed as mean (SEM). Results were compared using Student's *t* test for normally distributed data. Non-parametric data were compared using the Mann-Whitney *U* test or the Kruskal-Wallis test if more than two groups were compared. Linear regression or the Spearman *r* test was used to assess correlation. The level of significance was taken as $p<0.05$.

Results

RELEASE OF TNF-α BY ORGAN CULTURES

Concentrations of TNF-α measured in the supernatant fluid of control mucosa were very low (15·7 (5·4) pg/mg tissue) compared with affected Crohn's disease mucosa (62·3 (11·8) pg/mg tissue) ($p<0.001$; Fig 1). Non-affected mucosa samples from patients with Crohn's disease produced significantly higher amounts of TNF-α (39·4 (9·7) pg/mg of tissue; $p<0.03$) than those of controls. On the other hand the amounts were not significantly different from affected samples from patients with Crohn's disease ($p=0.14$; Fig 1). However, for patients in whom biopsy specimens were taken from both affected and non-affected mucosa (n=17), TNF-α released by affected areas significantly exceeded that released by normal appearing areas (Fig 2; $p<0.03$).

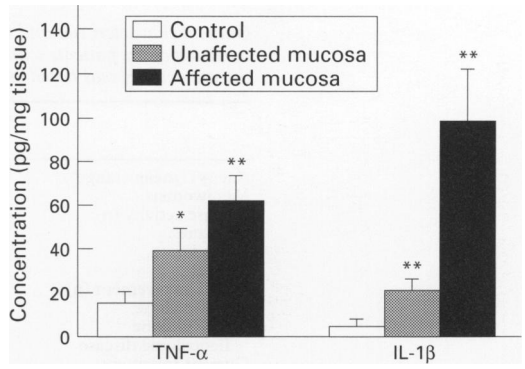

*Figure 1: Spontaneous release of TNF-α and IL-1β from endoscopic biopsies of normal intestine (control; n=26), unaffected (endoscopically and histologically) (n=26) and affected (n=26) mucosa of patients with Crohn's disease maintained in organ cultures for 18 hours. Results are expressed as mean (SEM) (*p<0·05; **p<0·01 v control). IL-1β release was significantly lower in unaffected than in affected mucosa (p<0·004) whereas TNF-α release was high in the two groups (p=0·14).*

RELEASE OF IL-1β BY ORGAN CULTURES

Supernatants of 19 control biopsy specimens had no detectable IL-1β. The seven remaining control intestinal biopsies spontaneously released small amounts of IL-1β (5·6 (2·5) pg/mg tissue). Spontaneous secretion of IL-1β by affected mucosa obtained from patients with Crohn's disease (99·8 (23·2) pg/mg tissue) was much increased (Fig 1; $p<0.002$). Non-affected intestinal biopsy specimens from patients with Crohn's disease released significantly higher IL-1β concentrations (21·5 (5·4) pg/mg tissue) compared with controls ($p<0.008$). These concentrations were lower than those of biopsies from affected mucosa ($p<0.004$) in patients with Crohn's disease (Fig 1). When biopsies were collected in both inflamed and macroscopically normal mucosa in the same patients (n=17) IL-1β was significantly higher in affected areas (Fig 2; $p<0.01$).

RELEASE OF IL-6 BY ORGAN CULTURES

Release of IL-6 was found in supernatants of cultured intestinal biopsy specimens obtained from controls (112·7 (11·5) pg/mg tissue). For affected Crohn's disease biopsy specimens IL-6 concentrations were significantly higher (1511·5 (451·5) pg/mg tissue) than samples from control mucosa (Fig 3; $p<0.004$). Biopsy specimens from non-affected area produced significantly smaller amounts of IL-6 (286·2 (55·5) pg/mg tissue) than those secreted by affected Crohn's disease tissue (Fig 3; $p<0.01$). However, these concentrations were significantly higher than those of controls ($p=0.005$). When biopsies were performed in the same patient in both affected and normal appearing mucosa, IL-6 concentrations were higher in

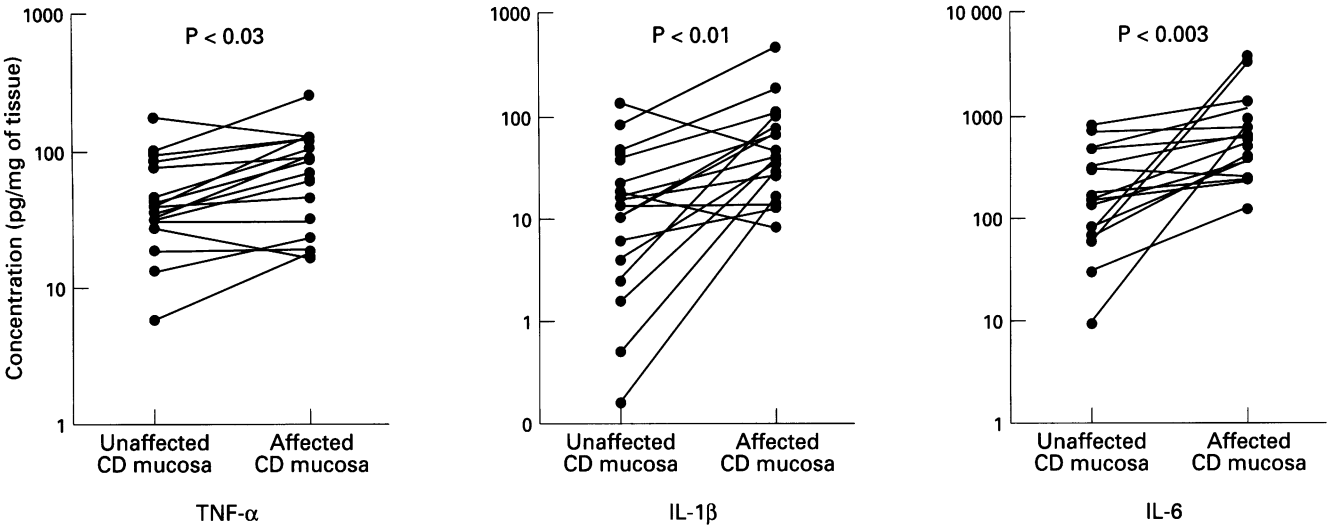

Figure 2: Individual concentrations of TNF-α, IL-1β, and IL-6 (log scale) in the culture supernatant fluids of intestinal mucosa from patients with Crohn's disease (n=17): biopsy specimens from both normal appearing (unaffected) and inflamed (affected) areas in the same patient.

the supernatants of organ cultures of inflamed tissue (Fig 2; p<0·003).

CORRELATION OF CYTOKINE RELEASE TO ENDOSCOPIC GRADE

IL-1β and IL-6 secretions were different in the three groups defined by endoscopic grading (respectively p<0·01 and p<0·001, Kruskal-Wallis) with a positive correlation for endoscopic grade (Spearman r=0·61, p<0·001 for both IL-1β and IL-6). Secretion of TNF-α was not different according to the endoscopic score (p=0·13).

CORRELATION OF CYTOKINE RELEASE WITH HISTOLOGICAL GRADE

Concentrations of IL-1β and IL-6 had a high correlation with histological assessment (Spearman r=0·62, p<0·001 for both). By contrast, TNF-α did not show any correlation with histological grading (r=0·19, p=0·21).

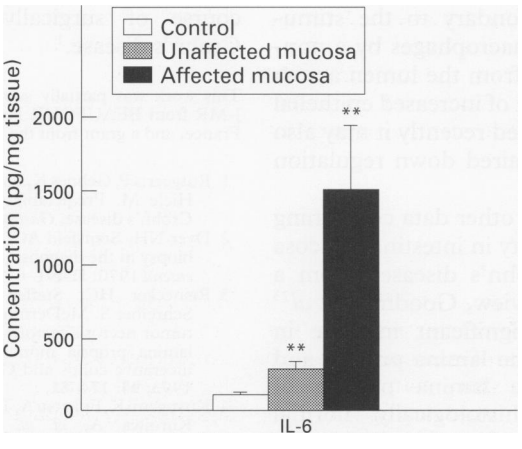

*Figure 3: Spontaneous release of IL-6 from biopsies of normal intestine (control; n=26), unaffected (endoscopically and histologically) (n=26), and affected (n=26) mucosa of patients with Crohn's disease maintained in organ cultures for 18 hours. Results are expressed as mean (SEM) (**p<0·01 v control). IL-6 release was significantly lower in unaffected than affected Crohn's disease mucosa (p<0·01).*

CORRELATION BETWEEN CYTOKINES

Concentrations of IL-1β and IL-6 correlated positively with each other (r=0·58, p<0·001). There was a weak correlation between IL-1β and TNF-α concentrations (r=0·43, p<0·005) and no correlation between TNF-α and IL-6 concentrations (r=0·15, p<0·3).

RELATION WITH OTHER VARIABLES

Cytokine production by mucosal samples were not correlated to disease activity or duration, location of bowel involvement, previous surgery, or type and duration of medical treatment (data not shown).

Discussion

In the present study we documented a significant inflammatory cytokine release by endoscopically and histologically normal mucosa of patients with Crohn's disease: IL-6, IL-1β, and mainly TNF-α concentrations were significantly higher in supernatant fluids from non-affected Crohn's disease mucosa than in controls. This detection of high concentrations of these cytokines by endoscopically and histologically normal mucosa strongly suggests their involvement in the early step of intestinal lesions in Crohn's disease. We also confirmed that the spontaneous release of TNF-α, IL-1β, and IL-6 by affected Crohn's disease mucosa was significantly higher than their release by control biopsy specimens.[12–14]

Concentrations of TNF-α were higher in the supernatant fluid from both inflamed and non-inflamed Crohn's disease mucosa than in controls, with a slight increase in TNF-α release by affected compared with non-affected Crohn's disease intestine and lack of correlation with the histological index. The pathogenic role of TNF-α in Crohn's disease has been controversial for a long time as a result of contrasting studies about this cytokine in inflammatory bowel disease. There were discrepancies between authors who investigated TNF-α mRNA by the polymerase chain

reaction and did not find increased concentrations of the transcripts in Crohn's disease tissue,[15 16] and studies in which mucosal immunoreactivity for TNF-α was measured showing an increased submucosal perivascular immunoreactivity related to recruitment of TNF-α secreting cells.[17] The second group of studies were reinforced by the finding of raised TNF-α concentrations in the supernatant fluids of organ cultures of affected mucosa from patients with Crohn's disease[5 12] compared with normal controls (as confirmed in the current study), as well as finding TNF-α mRNA expressing cells in the tissue of patients with Crohn's disease.[18] These results are in accordance with the recent report of the therapeutic efficacy of chimeric anti-TNF-α monoclonal antibodies in 10 patients with Crohn's disease refractory to steroids.[19] Taken together, these previous studies and the present one strongly support the idea that TNF-α is of major importance in the pathogenesis of Crohn's disease.

Our data concerning TNF-α production by non-affected mucosa of patients with Crohn's disease contrast with those of Jones *et al.*[12] However, they are in accordance with the findings of Breese *et al*,[17] who reported that TNF-α secreting cells were increased in patients with minor histological abnormalities and also showed a lack of a drug effect (except cyclosporine) on TNF-α immunoreactivity. Furthermore, raised TNF-α concentrations were recently described by Peeters *et al*[20] in the supernatant fluid of macroscopically normal ileum and colon from patients with Crohn's disease.

Raised IL-1β and IL-6 productions by affected tissue is universally recognised,[12–14] but the finding of enhanced release of IL-1β and IL-6 release by non-affected Crohn's disease mucosa is more exciting. These results are in accordance with the recent data of Scher *et al*[21] and Peeters *et al.*[20] By contrast with TNF-α production, we found a highly positive correlation between IL-1β and IL-6 concentrations and histological inflammatory grade.

This chronic production of inflammatory cytokines could be secondary to the stimulation of subepithelial macrophages by a non-specific antigen leaking from the lumen across the epithelium as a result of increased epithelial permeability. As suggested recently it may also be the result of an impaired down regulation of cytokine secretions.[22]

Our results fit in with other data concerning the questions of normality in intestinal mucosa from patients with Crohn's disease. From a morphometric point of view, Goodman *et al*[23] first demonstrated a significant increase in plasma cell density in the lamina propria and in the volume of the lamina propria of macroscopically and histologically normal appearing rectal mucosa from patients with Crohn's disease. This study was reinforced from a biochemical point of view by Dunne *et al*[10] who found, in addition to an increase in mucosal volume, a significant decrease in disaccharidase activity by apparently safe upper jejunal mucosa from patients with Crohn's disease. More recently evidence has been produced of enhanced complement secretion by unaffected jejunal tissue from patients with Crohn's disease,[24] and increased phospholipase A_2 activity.[25] Our study provides further evidence to support this concept of diffuse involvement of the whole intestinal tract in Crohn's disease. In addition, Nagel *et al*,[26] in accordance with previous reports,[27] recently described early epithelial lesions in apparently unaffected mucosa from patients with Crohn's disease, detected by scanning electron microscopy but not by conventional light microscopic examination; the light microscope appearing to underscore mucosal integrity. Furthermore, it is interesting to keep in mind that the pathological figures detected by these ultrastructural investigations may correspond to those observed in early apoptosis and to remember that cytokines participate in these phenomenon of programmed cell death.[28]

The present results point out that mediators involved in the initial steps of inflammatory/immune response (inflammatory cytokines) and known to induce cell death may be present in the normal appearing mucosa from patients with Crohn's disease. These cytokines represent potential targets of pharmacological intervention and therefore, the development of inflammatory cytokine inhibiting agents, not only for the treatment of acute relapses but also for maintenance treatment.

Taken together with recent medical literature, our results shed a new light on the potential importance of inflammatory cytokines in the perpetuation of the intestinal inflammatory response in Crohn's disease. They provide evidence for a persisting immune stimulation even in the absence of demonstrable endoscopic or histological inflammation. Furthermore, the presence of inflammatory cytokines - in particular of TNF-α – together with other lymphokines in the normal appearing mucosa from patients with Crohn's disease[29] suggest some explanation for the frequency of granulomas in otherwise endoscopically and histologically normal Crohn's disease mucosa,[2 30] and, may be, the high recurrence rate in the postoperative course of surgically treated patients with Crohn's disease.[1]

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